A sex-specific marker for shrimps and prawns

The present invention relates to a sex-specific marker for shrimps and prawns. More specifically, it relates to a sex-specific PCR-based molecular marker, derived from *Penaeus monodon*, that can be used to determine the sex in shrimps and prawns and can be used for any and all requirement that require the determination of genetic sex in shrimp and prawn including but not limited to sex determination of very young animals, determination of genetic sex on any animals and setting up monosex cultures.

Shrimp and prawn cultivation and trade is a very important activity all over the world. The main species under cultivation are *Penaeus monodon* (Giant tiger prawn, Jumbo tiger prawn, Jumbo tiger shrimp, Black tiger prawn, Blue tiger prawn, Grass shrimp…), mainly cultivated in Asia, with an aquaculture production of about 600,000 tonnes in 2003; and *Penaeus vannamei* (Whiteleg shrimp, white shrimp), mainly cultivated in the Americas and in China and Thailand, with an aquaculture production that is comparable to *P. monodon.* For those species, aquaculture is far more important than capture.

Increasing demands for aquaculture production mean increasing pressure for the development of more efficient production systems. More and more, modern genetics are used to support stock improvement and breeding programmes (Hulata, 2001). Genomic research and gene mapping developed fast during recent times. DNA markers have been characterized for use in establishing pedigrees, linkage mapping and identifying Quantitative Trait Loci (QTLs).

As most *Penaeus* sp. are sexually dimorphic (Hansford and Hewitt, 1994), a lot of effort has been made to find a reliable sex marker, which could help in setting up and maintaining monosex cultures. Several groups developed linkage maps, mainly based on the use of AFLP markers (Moore *et al.*, 1999; Wilson *et al.*, 2002). Pérez *et al.* (2004) published a sex-specific linkage map of the white shrimp *Penaeus vannamei*. However, they did not identify a sex-linked marker or linkage group. Li *et al.* (2003) disclosed a sex-specific linkage map of *Penaeus japonicus*, with a presumed sex marker on the maternal linkage map. Zhang et al. (2006) published a linkage map of P. vannamei, with sex-linked microsatelitte markers present on the female map. In the latter two cases, however, the sex-marker association was not challenged among genetically unrelated individuals. It should be stressed that the relatively low number of sample meioses within designed populations (e.g. half-sib families) leads to relatively long stretches of chromosomes being in Linkage Disequilibrium (LD). Consequently, in such linkage studies, the observed high LD between marker and sex dimorphism results from the nature of the population rather than from the tight physical linkage. Therefore, markers found via linkage analysis to be in LD with the sex often fail to discriminate between the two sexes in a population of unrelated individuals. Indeed, Li *et al.* (2003) admit that the presumed marker is not necessarily linked to a sex-specific sequence, and no sequence data are disclosed. Moreover, Zhang *et al.* (2004) were unable to identify sex-specific markers in

Penaeus chiniensis using the AFLP approach. Likewise, Khamnamtong *et al.* (2006) could not identify sex-specific markers in *Penaeus monodon*.

Surprisingly, using AFLP technology, we were able to isolate a prawn or shrimp sex-specific sequence, allowing unambiguous sex determination of both males and females. To identify sex-specific markers a large-scale bulked segregant analysis (BSA) using AFLP technology was performed in *Penaeus monodon*. An initial screening was performed in one experimental mapping population. Candidate sex-specific markers were confirmed in three additional experimental mapping populations and in a large set of unrelated wild-caught adults. Two markers were consistently sex-specific at all of these stages. One of these two AFLP markers was subsequently converted to a PCR-based co-dominant marker.

A first aspect of the invention is a prawn or shrimp sex-specific sequence. Sex-specific sequence, as used here, means that the sequence can be used for unambiguous sex discrimination between males and females. Preferably, said sex-specific sequence is limited in length, to allow an easy identification of small differences between male and female sequences. More preferably, said sex-specific sequence is not more than 2000 nucleotides in length, even more preferably not more than 1000 nucleotides, even more preferably not more than 500 nucleotides, even more preferably not more than 400 nucleotides in length. Most preferably said sex-specific sequence is comprising SEQ ID N° 3 and/or SEQ ID N°4, or a functional fragment thereof. One preferred embodiment is a female specific sequence consisting of SEQ ID N°3. Another preferred embodiment is a sequence consisting of SEQ ID N° 4 for which males are homozygous. A functional fragment, as used here, is a fragment carrying a sex-specific single nucleotide polymorphism (SNP) and/or an insertion deletion (INDEL), and/or a fragment that allows amplification of such sex-specific SNP and/or INDEL. As a non-limiting example, said specific fragment comprises a SNP situated at position 106, 121, 161, 191, 198 and/or 291 of SEQ ID N° 3, and/or and INDEL situated at position 62, 111-117, 216 and/or 272 of SEQ ID N° 3. Preferably, said functional fragment is a primer comprising, preferably consisting of SEQ ID N°1 or SEQ ID N° 2. Said primer can be used to amplify the sex-specific INDEL situated at position 111-117 of SEQ ID N° 3. Preferably said prawn or shrimp belongs to the family of the *Penaeidae*. Even more preferably, said prawn or shrimp is a *Penaeus* sp., including, but not limited to *P. monodon, P. vannamei, P. japonicus, P. indicus, P. merguiensis, P. schmitti* and *P. stylirostris.* Most preferably, said prawn or shrimp is *P. monodon.*

Another aspect of the invention is the use of a PCR-based marker to determine the sex in prawns and shrimps. PCR-based marker, as used here, can be any nucleic acid sequence that upon PCR amplification, allows an unambiguous determination of the sex. Preferably, said marker is limited in length, to allow an easy identification of small deletions. More preferably, said marker is not more than 2000 nucleotides in length, even more preferably not more than

1000 nucleotides, even more preferably not more than 500 nucleotides, most preferably not more than 400 nucleotides in length. Nucleic acid sequence as used here may be any nucleic acid sequence, including but not limited to DNA, cDNA and RNA. The PCR technology used may be any PCR-based technology known to the person skilled in the art. Preferably, the amplified sequence is selected from the group comprising, more preferably consisting of SEQ ID N° 3 and SEQ ID N° 4, or a functional fragment thereof, carrying a sex-specific SNP and/or INDEL. Preferably, said marker is amplified using primers selected from the group consisting of SEQ ID N° 1 and SEQ ID N° 2. Preferably said prawn of shrimp belongs to the family of the *Penaeidae*. Even more preferably, said prawn or shrimp is a *Penaeus* sp., including, but not limited to *P. monodon, P. vannamei, P. japonicus, P. indicus, P. merguiensis, P. schmitti* and *P. stylirostris.* Most preferably, said prawn or shrimp is *P. monodon.* Although the detection of the PCR-based marker is preferably done using a PCR-based technology, it is clear for the person skilled in the art that other technologies, such as, but not limited to DNA-DNA hybridization, micro-array technology or DNA melting profiles, alone or in combination with PCR amplification, can be used for the detection of the marker sequence.

Still another aspect of the invention is a method of setting up a monosex culture in prawns and shrimps, comprising a PCR-based sex determination according to the invention. The PCR-based sex determination can be used to distinguish between males and females, and select the organisms to be cultured. However, setting up a monosex culture, as used here, does not imply that the PCR-based sex determination according to the invention should be used in every culture cycle. Indeed, the PCR-based sex determination according to the invention may be used, as a non limiting example, in the selection of homogametic females, which, upon crossing with homogametic males, would give a completely uniform and heterogametic female offspring, resulting in a monosex culture. Preferably said method comprises the use of primers selected from the group consisting of SEQ ID N° 1 and SEQ ID N° 2. Even more preferably, the sequence, amplified by the PCR-based sex determination is selected from the group consisting of SEQ ID N° 3 and SEQ ID N° 4, or a functional fragment thereof, carrying a sex-specific SNP and/or INDEL. Preferably said prawn of shrimp belongs to the family of the *Penaeidae*. Even more preferably, said prawn or shrimp is a *Penaeus* sp., including, but not limited to *P. monodon, P. vannamei, P. japonicus, P. indicus, P. merguiensis, P. schmitti* and *P. stylirostris.* Most preferably, said prawn or shrimp is *P. monodon.*

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Genotyping of 52 broodstock animals using the INDEL-marker.

Figure 2: Schematic representation of the female-specific AFLP fragment (A) and PCR fragments used for obtaining the full sequence of this fragment (B-D).

Figure 3: Melting curves for RT-PCR amplified SNP for female and male *P. monodon*

EXAMPLES

Materials and Methods to the examples:

Animals

Four half-sib mapping populations were generated by crossing two female animals (IC100 and IC67) with two male animals (AL91 and AL99) in all four possible combinations (see Table 1).

Table 1: Parents and number of progeny of the four half-sib mapping populations used in this study.

Population	female	male	number of progeny	number of females	number of males	Number of doubts*
A	IC100	AL91	111	55	45	11
B	IC67	AL91	113	62	45	6
C	IC100	AL99	120	59	61	0
D	IC67	AL99	120	56	57	7

* The phenotypic sex of these individuals could not be unambiguously determined.

All four parents were recently caught in the wild. All crosses were performed at Moana Technologies, Inc. The mapping populations were harvested at post-larval stage (PL) 120, when the sex of each individual could be scored with an acceptable degree of certainty. In addition, a set of 52 unrelated wild-caught animals was available at Moana Technologies, Inc. These individuals were used to assess the tightness of the physical linkage between the sex-locus and the potential sex-markers identified in the mapping populations.

AFLP screening

DNA was prepared from snap-frozen pleopod tissue using a CTAB method optimised for shrimp tissue. AFLP analysis was performed as described by Vos *et al* (VOS *et al.* 1995). Genomic DNA of the parents and progeny was restricted using EcoRI and MseI. Double stranded adaptors were ligated to the ends of the restriction fragments. The digestion product was diluted and pre-amplified using adaptor-specific primers with a single selective nucleotide on each primer. Two pools, or bulks, of five individuals of population A were made at the pre-amplification level. Within each pool, or bulk, the individuals were identical for sex but arbitrary for all other loci. Selected subsets of restriction fragments were amplified using AFLP-primers

containing 2 additional selective nucleotides. Amplification reactions were separated on AFLP sequencing gels and visualised using LI-COR IR^2 technology.

AFLP markers identified as sex-specific in the Bulk Segregant Analysis (BSA) on population A (i.e. that were present in the two female bulks but not in the two male bulks) were considered candidate sex-specific markers. Subsequenlty, these candidate sex-specific markers were tested on the bulked samples from the three remaining mapping populations. Markers that confirmed their association to sex in the three additional BSA analyses were then tested for linkage to the sex-locus in each of the four mapping populations.

To finally assess the strength of the identified marker-trait-associations by linkage, we genotyped the set of 52 unrelated wild-caught (broodstock) animals (the four parents from the mapping populations and 48 additional samples) with the AFLP markers found to be in linkage with sex.

Sequencing

To obtain sequence information from AFLP markers, the EcoRI-specific primer was radioactively-labelled using ^{33}P-ATP and amplification products were separated on a 5% denaturing sequencing gel. Gels were dried and visualised by autoradiography. After visualization, the bands were excised from the gel, and eluted fragments were amplified and sequenced.

PCR amplification

Most PCR reactions were performed in 1xPCR buffer supplemented with 1.5 ng/µl of each primer, 0.2 mM of each dNTP, 2.5 mM of MgCl2, 0.025 U of Taq polymerase and 100 ng of template DNA.

PCR using primers ATTGCA-1 and ATTGCA-2 were performed in a total volume of 20 µl using 100 ng of genomic DNA as a template. The reaction mixture was heated to 95 ºC for 4 minutes, followed by 35 cycles of 30 seconds of denaturation at 95 ºC, 30 seconds of annealing at 52 ºC and 30 seconds of elongation at 72 ºC. Finally, an additional elongation step of 2 minutes at 72 ºC was performed.

PCRs using primer ATTGCA-1 in combination with primers MseI+GCA or MseI+AAA and PCR using primer ATTGCA-2 in combination with primer EcoRI+ATT were performed in a total volume of 50 µl using 5 µl of a 1/20 diluted preamplification reaction. PCR reactions were as follows: 35 cycles of 30 seconds of denaturation at 95 ºC, 30 seconds at the appropriate annealing temperature (55 ºC for ATTGCA-1 and 53 ºC for ATTGCA-2) and 30 seconds of elongation at 72 ºC followed by an additional elongation step of 2 minutes at 72 ºC.

PCR for the INDEL-marker were performed using either radioactively or fluorescently labelled primer INDEL-4. The reaction was performed in 1xPCR buffer supplemented with 0.3 ng/µl of

each primer, 0.2 mM of each dNTP, 2.5 mM of MgCl2, 0.025 U of Taq polymerase and 50 ng of genomic DNA. The reaction mixture was heated to 95 ºC for 4 minutes, followed by 35 cycles of 30 seconds of denaturation at 95 ºC, 30 seconds of annealing at 55 ºC and 30 seconds of elongation at 72 ºC. Finally, an additional elongation step of 2 minutes at 72 ºC was performed. For the fluorescent analysis, the reaction was essentially the same as for the radio-active analysis except that 0.03 mM of IRD700-labelled INDEL-4 primer was added and 0.3 mM of INDEL-5 primer. Primer sequences are given in table 2.

Table 2: Primer sequences used in this study.

Primer name	Primer sequence (5'-3')
EcoRI-specific AFLP primer	GAC TGC GTA CCA ATT C
MseI-specific AFLP primer	TGA GTC CTG AGT AA
ATTGCA-1	TCT AAC AGT TCA TAA AGC ATC CTA T
ATTGCA-2	TTA AGC ATA TAC TAA GAA TCC AT
INDEL-4	GGG GTC GCG AAT GTA AAA TA
INDEL-5	TTT TCA AAT GCA TAA CTG TTA GCT G

SNP genotyping

Genomic DNA was prepared from a small sample of tissue (e.g. 5 mm of the tip of a walking leg) by heating for 30 minutes at 95°C in 75 µl of alkaline lysis buffer (25 mM NaOH, 0.2 mM EDTA pH = 12). The samples were cooled on ice and 75 µl of neutralization buffer (40 mM Tris-HCl pH = 5) was added. PCR was performed on 5 µl of the 1/20 diluted DNA.

PCR was performed using the “Platinum sybr green qPCR supermix-UDG” kit (Invitrogen) using primers FemaleForward_2, MaleForward_2 and Reverse_2. An initial denaturation step (5 minutes 95°C) was followed by 40 cycles of 20 seconds 95°C, 30 seconds 55°C and 20 seconds 72°C. Reactions were subsequently denatured (1 min 95°C) and renatured (1 min 40°C). Melting curve analysis was performed on an iCycler system (Bio-Rad).

Primer sequences:

FemaleForward_2: GCGGGCGTTAGCTGATATTCATAATTCATGCTC

MaleForward_2: GCGGGCAGGGCGGCGTTAGCTGATATTCATAATCCATGCAA

Reverse_2 : AAGGGGTCGCGAATGTAAAATA

Example 1: Marker identification

In a first screening, we analysed approximately 1,408 AFLP primer combinations on the bulked samples from mapping population A. The AFLP EcoRI+3/MseI+3 primer combinations generate 50-80 AFLP fragments. Hence, the bulks were fingerprinted with more than 70,400 AFLP fragments. Of these fragments, 13 were identified by the BSA analysis as sex-specific. To confirm this, bulked samples from the other three mapping populations (B, C and D) were fingerprinted for these 13 sex-specific markers. In nine cases the marker-sex association could be confirmed. All of these markers were present in the female bulks but absent from the male bulks.

To determine how tightly these nine markers were linked to the sex-locus, markers were scored in all offspring from the four mapping populations. The recombination frequency (i.e. the number of females that did not show a band and the number of males that did show a band, expressed as a proportion of the total number of individuals analyzed) is expressed in Table 3.

Table 3: Recombination frequency between the sex-locus and each of the nine sex-linked AFLP markers in each of the four mapping populations.

MARKER (*)	POPA IC100xAL91	POPB IC67xAL91	POPC IC100xAL99	POPD IC67xAL99
E+AAG/M+CGC-72.8	0/100	0/96	0/120	0/109
E+ACC/M+GGG-183.8	0/99	0/89	0/115	0/111
E+AAC/M+TAG-200.7	1/99	6/94	2/118	4/110
E+AGA/M+CAG-333.0	0/99	1/92	0/117	1/108
E+CAG/M+GAG-148.2	0/98	0/97	0/116	0/112
E+ACT/M+GTC-284.4	0/98	0/98	0/119	0/112
E+AAA/M+GTG-125.4	0/98	0/91	0/119	0/112
E+CGG/M+TTG-489.3	1/96	5/92	0/119	0/111
E+ATT/M+GCA-347.0	0/100	0/96	0/120	0/109

(*) E: EcoRI; M: MseI - indicating the sequence of the specific primers according to table 2, extended with the selective nucleotides applied

To assess the degree of LD of the AFLP marker haplotypes with the sexual dimorphism in a population of genetically unrelated individuals, the four parental animals and 48 additional broodstock animals, recently caught in the wild at several locations throughout the Pacific Ocean were genotyped at the nine sex-linked AFLP marker loci. At two loci, AFLP marker alleles (EcoRI+AAG/MseI+CGC-72.8 and EcoRI+ATT/MseI+GCA-347.0) were in complete LD with the sex of *P.monodon*.

Example 2: Marker sequencing

The corresponding EcoRI-specific primers were radioactively labelled. The AFLP amplification products were separated on a denaturing polyacrylamide sequencing gel and visualised using autoradiography. The female EcoRI+AAG/MseI+CGC-72.8 and EcoRI+ATT/MseI+GCA-347.0 marker allele were cut from the AFLP gel, eluted, amplified and sequenced. Because we obtained multiple possible sequences, we increased the selectivity of the AFLP reaction by two additional selective nucleotides (+3/+3 AFLP reaction → +4/+4 AFLP reaction). The female-specific fragments were now obtained as EcoRI+AAGT/MseI+CGCT-72.8 and EcoRI+ATTA/MseI+GCAT-347.0. Isolation and subsequent sequencing of the EcoRI+ATTA/MseI+GCAT-347.0 marker band resulted in a unique sequence. A non-specific band hampered the isolation of the EcoRI+AAGT/MseI+CGCT-72.8 fragment and its subsequent generation of a unique sequence. Furthermore, the sequence was short, making it even more difficult to design a specific PCR for this fragment. Therefore, marker EcoRI+ATTA/MseI+GCAT-347.0 was chosen to further develop into a co-dominant PCR-marker.

For marker EcoRI+ATTA/MseI+GCAT-347.0 we designed PCR primers to amplify the marker in both female and male individuals. Using primers ATTGCA-1 and ATTGCA-2, we were able to amplify a fragment of approximately 285 base pairs (bp) on the genomic DNA of both female and male individuals. These PCR fragments were isolated from the gel and sequenced. This resulted in a female- and male-specific sequence for this marker. Careful examination of these sequences revealed nine sex-specific polymorphisms in these sequences: 6 single nucleotide polymorphisms (SNPs) and 3 Insertion/Deletion (INDEL) polymorphisms. One of the INDELs caused the presence of an additional MseI restriction site in the male. This is the polymorphism most probably responsible for the absence of the EcoRI+ATTA/MseI+GCAT-347.0 AFLP fragment in the males. To obtain sequence information of the part of flanking regions of the AFLP fragments, we performed PCR using primers ATTGCA-1 and the corresponding MseI-specific AFLP primer (M+GCA for the female and M+AAA for the male) and using primers ATTGCA-2 and the corresponding EcoRI-specific AFLP primer (EcoRI+ATT for both female and male) (see Figure 2). The resulting PCR fragments were sequenced and the previously obtained sequences were updated using this additional sequence information

(SEQ ID N° 3 and 4). As a result, an additional sequence polymorphism (INDEL) between the male and female sequence was detected which was previously not identified because it is located in the sequence targeted by primer ATTGCA-1.

Example 3: Conversion of AFLP marker E+ATTA/M+GCAT-347.0 to a PCR-based co-dominant INDEL-marker.

To convert the E+ATTA/M+GCAT-347.0 AFLP marker into a simple single locus marker, we designed primers to specifically amplify the genomic locus harbouring the INDEL polymorphism identified to be sex-specific. Using primers INDEL-4 and INDEL-5, an allelic fragment of 76 bp was amplified in five males and five females coming from population A, and an allelic fragment of 82 bp was amplified in the five females only. This proved that females are the heterogametic sex in *Penaeus monodon*. A similar result was obtained for the set of 52 unrelated broodstock animals, showing that the INDEL polymorphism is in complete LD with the sex (see Figure 1). Another set of 33 unrelated broodstock animals was genotyped with this marker and again the INDEL polymorphism was found to be in complete LD with the sex.

The PCR-amplified marker alleles described here are in complete LD with the sex dimorphism in *Penaeus monodon*. This marker allows the determination of the genetic sex of any *P. monodon* individual regardless of its developmental stage.

Example 4: development of an RT-PCR based SNP genotyping assay for sex in *P.monodon*.

To facilitate the screening of large numbers of shrimp, we developed an RT-PCR based SNP genotyping assay for the sex marker. Specific forward primers were designed for the male and the female allele and used in a PCR in combination with a common reverse primer. After amplification, melting curve analysis was performed. In males, only one peak was observed, while in the female two peaks corresponding to the two alleles, were observed (Figure 3).

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CLAIMS

1. A prawn or shrimp sex-specific sequence.
2. A prawn or shrimp sex-specific sequence, comprising SEQ ID N°3 or SEQ ID N° 4 or a functional fragment thereof.
3. A functional fragment of a prawn or shrimp sex-specific sequence according to claim 2, comprising SEQ ID N° 1 and/or SEQ ID N° 2.
4. A prawn or shrimp sex-specific sequence, or a functional fragment thereof according to any of the preceding claims, whereby said prawn or shrimp belongs to the family of *Penaeidae.*
5. A prawn or shrimp sex-specific sequence, or a functional fragment thereof, according to claim 4 whereby said prawn or shrimp is *Penaeus monodon.*
6. The use of a PCR-based marker to determine the sex in prawns and shrimps.
7. The use of a PCR-based marker according to claim 6, whereby the amplified sequence is selected from the group comprising of SEQ ID N°3 - SEQ ID N°4, or a functional fragment thereof.
8. The use of a PCR based marker according to claim 6 or 7, whereby the marker is amplified using primers selected from the group consisting of SEQ ID N° 1 - SEQ ID N° 2.
9. The use of a PCR-based marker according to any of the claims 6-8, whereby said prawn or shrimp is a *Penaeus* sp.
10. The use of a PCR-based marker according to claim 9, whereby said prawn or shrimp is *Penaeus monodon.*
11. A method of setting up a monosex culture in prawns or shrimps, comprising a PCR-based sex determination.
12. The method according claim 11, whereby said PCR-based sex determination comprises the use of primers, selected from the group consisting of SEQ ID N°1 - SEQ ID N° 2.
13. The method according to claim 11 or 12, whereby said PCR-based sex determination comprises the use of a sequence selected from the group consisting of SEQ ID N°3 - SEQ ID N°4, or a functional fragment thereof.
14. A method according to any of the claims 11-13, whereby said prawn of shrimp is a *Penaeus* sp.
15. The method according to claim 14, whereby said prawn or shrimp is *Penaeus monodon.*